Detection of Citrus Greening Bacterium by Immunoblotting

Ratana Sdoodee¹ and Helen Garnett²

Abstract
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Polyclonal antibodies raised against cultured African greening bacterium were successfully used in immunoblotting assays for detection of Thai greening. The assays were made in crude sap of citrus midribs collected from suspected diseased trees throughout Thailand. The results of immunoblotting tests indicated that the antisera were specific to Thai greening, as 130/190 Som Khieo Waan (Siam mandarin), 21/23 Shogun (Shogun mandarin), 30/139 Som Chuk (Neck orange), 12/27 pomeloes, and 9/9 Som Cheet (calamondin) gave positive reactions, while none of the healthy controls did. The incidence of greening in the tested trees showing reactions with greening antisera was confirmed by electron microscopy indicating the presence of pleomorphic greening bacterium-like organisms in their phloem cells. Transmission of putative greening from positive citrus trees by insect vector (Diaphorina citri Kuwayama) to healthy index plants was attempted to support results from immunoblotting assays. The greenings were transmitted from 4/4 Som Chuk and 6/6 Shogun.

Key words: greening bacterium, citrus, detection, immunoblotting.

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Received, September 1994
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Greening disease is a major problem for the citrus industry in 17 Asian and 20 African countries.\(^2\) It is responsible for a considerable loss of citrus trees every year, especially in nurseries and young orchards. Before it was identified as a disease, it was known by various names: yellow shoot (huanglungbin) in China; likubin (Decline) in Taiwan; dieback in India; leaf mottle in Philippines; vein phloem degeneration in Indonesia; and greening in South Africa and Thailand.\(^6,8,19\) There are two types of greening, African and Asian forms. They can be distinguished on the basis of temperature tolerance, as symptoms of the Asian and African greening appear at 22-38 °C and 22-24 °C, respectively.\(^6\) A diseased tree produces yellowing leaves along the veins and sometimes a blotchy - mottle coloring.\(^1\) Secondary symptoms are leaves which are small, upright, and show chlorotic patterns resembling those induced by zinc and iron deficiencies.\(^6\) Infected fruits are small, lopsided, have a bitter taste, and remain green on the shaded side.\(^17\) The Asian greening caused more extensive dieback and decline than the African. Both types of greening are reported graft-transmissible.\(^8\) Transmissions by insect vector have also been investigated; in citrus psylla species, Triozia erytreae transmitted African greening while Diaphorina citri was the vector of the Asian type.\(^8\)

Earlier, biological indexing by graft-transmission and electron microscopy (EM) were the only two diagnostic methods widely used. Transmission by grafting to healthy plants has been reported as being inconsistent.\(^10\) Subsequently, it was shown that the larger the graft or the greater number of buds, the greater the success rate achieved.\(^17\) Similarly, detection of greening bacterium by EM is unreliable because of uneven distribution of the pathogen in an infected tree.\(^15\) Rapid, reliable and sensitive procedures for the detection of greening bacterium, the causal agent, are needed to assist epidemiological studies and provide useful information for disease control. However, the bacterium's resistance to culture on artificial media has made study of the organism difficult.

Recently, nucleic acid probes and serological tests have been developed by various groups of plant pathologists.\(^1,5,12,14,21,22\) In France, DNA probes have
been prepared from cloned DNA from infected periwinkles, and from greening organism cells trapped by immunoaffinity chromatography, while development of probes from the cultured putative greening organism has begun in Australia. Monoclonal antibodies against several Asian and African isolates have now been raised by a French research group; in combination, these may have potentially universal use. Garnett has claimed to have isolated and cultured African greening bacterium. Subsequently, polyclonal antibodies against the cultured bacterium were produced. The antisera are able to detect the greening organism in the infected plant tissue by ELISA and are highly specific to the bacterium associated with greening disease when tested by immunogold-staining. However, they failed to react with greening infected tissue in tests by Garnier et al.

The experiment reported here was constructed to test the applicability of the antisera prepared from the cultured greening bacteria for detecting Thai greening using immunoblotting technique or Dot-ELISA.

Materials and Methods

Citrus samples
Several hundred samples of citrus were collected from greening affected areas throughout Thailand, including Chiang Rai (North), Pathum Thani (Central Plain), Chanthaburi (East), and Yala and Songkhla (South). Citrus seedlings grown in insect-proof glasshouse were used as healthy controls.

Greening Antisera
Polyclonal antisera raised in rabbits against the cultured African greening bacteria provided by Professor H. Garnett of Wollongong University, Australia, were used in all tests. Gamma-globulin (Ig G) was purified from the antisera by protein A affinity chromatography.

Immunoblotting assay
Citrus midriffs (0.5 g), either from collected samples or healthy leaves, were homogenized in phosphate buffer saline (PBS) at 1:10 (w/v). The homogenates were filtered through a single layer of Miracloth, diluted to 1:5 with PBS and then spotted onto a nitrocellulose membrane (NCM), MSNITROBIND Transfer Membrane, with 0.22 μm pore size, using a Bio-Rad Bio Spot Apparatus. The membrane was allowed to dry for 16-20 hrs. and then blocked with 3% gelatin in tris buffered saline (TBS) for 1 hr. before washing 2 times for 5 mins. per time in tween-tris buffered saline (TTBS). Following the washing stage, the NCM was gently shaken in 1 μg ml⁻³ greening IgG in 1% gelatin in TBS for 12-15 hrs. The NCM was washed in TTBS as previously mentioned and then transferred to goat anti-rabbit alkaline phosphatase conjugate (GAR-AP) diluted to 1:7,500 in 1% gelatin. It was shaken for 4 hrs. and subsequently washed in TTBS for 15 mins. followed by another 2 washes of TTBS and 2 washes in TBS for 5 mins. per time. A colour development solution (1.65 mg nitroblue tetrazolium, NBT, and 0.85 mg 5-bromo-4 chloro-3 indolylphosphate, BCIP, in 10 ml TBS) was added. After soaking in the colour development solution for 30 mins., the NCM was transferred to distilled water in order to stop the reaction. Schematic representation of the immunoblotting system is shown in Fig 1.

Electron microscopy
Small blocks of citrus midriffs were fixed, embedded in L.R. White resin and cut into ultrathin sections by Sorvall MT 5000 Microtome using a procedure described by Sandee. The sections were examined for greening by JEOL 100 CX II transmission electron microscope.

Insect transmission
Ten to 15 Diaportha citri Kuwuyama, trapped from citrus trees giving positive reaction in immunoblotting test, were allowed to feed on healthy Som Khieo Waan (Citrus reticulata Blanco), a greening index plant, for 7 days. The index plants were sprayed with insecticide and kept in insect-proof glasshouses at 25-32°C for 3 months before being assayed for greening by the immunoblotting method.
Results

Immunoblotting test

Preliminary tests indicated that greening assays by immunoblotting were readily achieved when NCM was incubated in 1 μg ml⁻¹ of Ig G and 20 μl of crude sap was applied. At this proportion, no background was detected and a positive dot was easily distinguishable from a negative one by visual assessment (Fig 2). The Ig G at the rate of 5 μg ml⁻¹ was also tested but the detectability reaction did not improve; there was a high background caused by the development of a pink coloration on NCM between the dots. When 10 or 15 μl of crude sap of infected sample was spotted for each dot, greening was not detected, despite a positive reaction being obtained from the dot of 20 μl of the same sample. With a positive reaction, a purple ring developed around the dot of crude sap, while with cultured greening bacterium (a positive control), the coloration occurred within the dot (Fig 2). In contrast, the sap from healthy citrus and noninfectious samples remained green (Fig 2).

Specificity of cultured African greening antisera to the Thai greening

The antisera were tested against citrus samples collected from Chiang Rai, Phathum Thani, Chanthaburi, Songkhla and Yala. Three species of citrus: *Citrus reticulata* Blanco including Som Khieo Waan (Siam Mandarin), Som Shogun, Som Chuk (Neck Orange); *C. maxima* (Burm.) Merill., pomeloes; and *C. mittis* Blanco, Som Cheet (calamondin) were examined. Greening organisms were specifically detected in 181/352 mandarins, 12/27 pomeloes and 9/9 calamonds (Table 1). The organisms were found in samples from all collected areas (Table 1). Moreover, most positive materials showed symptoms similar to those caused by greening disease (Fig 3).

Electron microscopy (EM)

Ultrathin sections of midrubs taken from Shogun trees giving positive reaction to the greening antisera were also examined by EM. The sections from the three positive Shogun plants revealed

<table>
<thead>
<tr>
<th>Citrus</th>
<th>Location</th>
<th>Incidence of greening</th>
<th>Total tested plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Thailand)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Siam mandarin</td>
<td>Yala (South)</td>
<td>89</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Phathum Thani (Central Plain)</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Chanthaburi (East)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Chiang Rai (North)</td>
<td>31</td>
<td>3</td>
</tr>
<tr>
<td>Shogun mandarin</td>
<td>Songkhla (South)</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>Neck orange</td>
<td>Songkhla (South)</td>
<td>30</td>
<td>109</td>
</tr>
<tr>
<td>Pomeloes</td>
<td>Songkhla (South)</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Chanthaburi (East)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Calamondin</td>
<td>Chanthaburi (East)</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>202</td>
<td>187</td>
</tr>
</tbody>
</table>

a Ig G of cultured greening antisera was previously cross absorbed with healthy citrus and purified Citrus Tristeza Virus (OD₂₆₀ = 0.15). The absorbed Ig G and GAR-AP were used at 1 μg/ml⁻¹ and 1 : 7500 respectively. A total of 22 healthy citrus plants were tested but none reacted with the antisera.

b + positive reaction
- negative reaction
Fig. 1 Schematic representation of the immunoblotting system.
1. Gelatin blocks unoccupied sites on nitrocellulose membrane.
2. Primary antibody specific to a greening antigen (produced in rabbit).
3. A blotting grade antibody enzyme conjugate (Goat anti-rabbit IgG-alkaline phosphatase, GAR-AP).

Positive samples

Negative samples

Healthy citrus

PBS

Cultured greening bacterium

Fig. 2 Colour development on nitrocellulose membrane of immunoblotting test.
pleomorphic greening bacterium-like organisms in their phloem cells (Fig 4). However, none of the healthy Shogun sections processed at the same time showed similar results. Previously, midribs from immunoblotting - unreacted trees: ten Som Chuk and five Shogun were also examined by EM for the presence of greening. However, the greening was not found in any of the fifteen samples.

Insect transmission

Transmissions of greening bacterium from the tested citrus trees to healthy index plants using insect vector (Diaphorina citri Kuwayama) were attempted to support the results from immunoblotting assays. It was found that greening bacteria from ten positive citrus trees were successfully transmitted by the vector (Table 2), as the index plants developed
mottled leaves and veinal chlorosis. Moreover, the index plants reacted positively to the greening antisera when tested by immunoblotting.

**Discussion**

In this experiment, polyclonal antibodies raised against the cultured African greening bacteria were successfully used for assaying Thai greening in the crude sap, using the immunoblotting technique. Similar results were obtained in all collected areas in term of infectious and non-infections materials (Table 1). In all tests, healthy citrus of the same species as collected samples served as control which were also examined to assure the nonspecific reaction. None of the healthy control responded to the antisera. The incidence of greening was high in the North, Central plain and East (Table 1) which is similar to results reported by Broadbent. In the South,
greening occurred in 45% of samples tested. Subsequently, electron microscopy (EM) confirmed that putative greening pathogens were actually present in the phloem tissue giving a positive reaction in immunoblotting tests (Fig 4). Although a few positive citrus samples were verified by EM, a hundred percent of materials revealed greening pathogen. Meanwhile, the pathogen was not found in the sections of either immunoblotting - unreacted citrus or healthy trees. Moreover the pathogen detected was shown to be readily transmitted by *D. citri* (Table 2) as is the insect vector of Asian type of greening.\(^{(3,7,9)}\)

It is more likely that the antisera against the cultured greening bacteria are specific to greening organisms existing in Thailand, as suggested by results from immunoblotting tests, electron microscopy and insect transmission; however, previous reports have suggested that the specificity of these antisera to the greening organism is erratic.\(^{(1,8,9)}\)

In other reports, monoclonal antibodies (MCA) raised against Indian and African greening could recognize greening isolate from India, the Philippines, and Reunion,\(^{(13)}\) but not ones from China,\(^{(10)}\) Thailand, Malaysia or other parts of India,\(^{(8)}\) while the MCA against Chinese isolate did not react with Indian greening.\(^{(8)}\) Currently, nucleic probes have been developed and more new accurate DNA probes were presented.\(^{(12)}\) The DNA probes were reported to respond with all strains of the Asian greening from China, India, Indonesia, the Philippines, Malaysia, Taiwan, Thailand and Vietnam.\(^{(22,23)}\) It is likely that the MCA are less effective than the DNA probes in detection of the Asian greening organisms. However, these probes were not able to recognize any south African strains of greening.\(^{(22,23)}\) In contrast, our results indicated that the polyclonal antibodies against African greening could cross react with the Asian type. Therefore, further testing of its performance should be made in comparison with the DNA probes and other new methods.

In addition, the immunoblotting technique or dot - ELLSA performed well in greening assay: positive reaction produced a bright stain (Fig 2) that contrasts sharply with the light-coloured NCM. Visual detection of the reaction is possible; unless quantitative measurement of reflectance among dots of different intensities are needed, photometric measurements appear unnecessary. This is a similar situation to the detection of potato virus S, X and Y by dot-ELISA on nitrocellulose membrane described by Banttari.\(^{(4)}\)

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**Table 2. Transmission of greening organism (GO) by *Diaphorina citri* Kuwayama from positive citrus to healthy Slam mandarin (index plant).**

<table>
<thead>
<tr>
<th>GO sources</th>
<th>Positive citrus sample code</th>
<th>Number of transmission per total index plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neck orange</td>
<td>1-1</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>9-2</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>9-3</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>10-2</td>
<td>2/3</td>
</tr>
<tr>
<td>Shogun mandarin</td>
<td>G 1</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>G 2</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>G 7</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>H 7</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>I 7</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>H 8</td>
<td>2/3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>24/30</strong></td>
</tr>
</tbody>
</table>
Acknowledgement

We wish to thank Australian Center for International Agricultural Research (ACIAR) for financial support and also to Ms. Saiyud Pukdeesuwon and Ms. Supaporn Kultoun for their technical assistant.

References


